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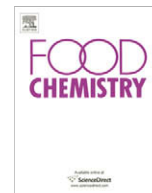

Production of D-galactose using β-galactosidase and *Saccharomyces cerevisiae* entrapped in poly(vinylalcohol) hydrogel

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ABSTRACT

D-Galactose was produced from lactose (200 g l^{-1}) in the batch mode of a simultaneous saccharification and fermentation process (SSF). β-Galactosidases (from *Kluyveromyces lactis* and *Aspergillus oryzae*) and yeasts were immobilized in poly(vinylalcohol) hydrogel lens-shaped capsules – LentiKats®. After 20 repeated batch runs with entrapped *K. lactis*, β-galactosidase and free *Saccharomyces cerevisiae* (10% v/v inoculum), galactose productivity decreased to 50% and 1.4 kg of galactose were prepared. Compared to this, just 20% decrease of galactose productivity and a 0.9 kg production of galactose were observed for the SSF process with β-galactosidase from *A. oryzae* after 15 repeated batches under the same conditions. In the process of SSF with co-immobilized enzyme from *K. lactis* and *S. cerevisiae*, the galactose productivity increased from 3 g l^{-1} h^{-1} to 4.1 g l^{-1} h^{-1}, which reduced the time of preparation of D-galactose.

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1. Introduction

D-Galactose, a monosaccharide found mostly in milk, has many applications, e.g. as pharmaceutical intermediate for several medicines, or as a stabilizer in intravenous solution for medical use. In the food industry, is widely used as a raw material for the manufacture of many sweeteners, such as arabinose, pentitol, arabinitol, globotriose, arbitol, galactitol, xylitol and tagatose (Granstrom, Takata, Tokuda, & Izumori, 2004; Ibrahim & Spradlin, 2000). Galactose is also useful in cell culture media as a nutrient or as an inducer in fermentations. Moreover, it can be used in the beverage industry, e.g. in sport drinks, in the manufacturing of contrast agents or as a substitute for phenols in resins (Darge, 2008; Ohlerth & O'Brien, 2007; Stannard, Hawke, & Schnell, 2007).

D-Galactose can be prepared from vegetable raw materials containing homologous (e.g. galactan) or non-homologous galactose polymers (e.g. legume material) (Molle & Kempener, 2000). The most common source of D-galactose is lactose (milk sugar), which is available cheaply and in large quantities as a by-product of the dairy industry. The process involves hydrolysis of lactose by enzymatic (Clyne & Wright, 1999) or chemical hydrolysis (Rosenberg, 2000), separation and purification of galactose. Galactose can be separated from a mixture of galactose and glucose by selective fermentation of the glucose to ethanol, using various kind of microorganisms (Cipolletti et al., 2007), or by glucose oxidase transformation of glucose into gluconic acid (Clyne & Wright, 1999).

To reduce the cost of D-galactose preparation, yeasts and enzymes were immobilized to poly(vinylalcohol) (PVA) gel particles LentiKats® in this study. Due to their shape (lenticular, diameter 3–4 mm, thickness 200–400 μm), LentiKats® have several advantages, e.g. superior mass transfer properties and easy separation from broth by sieves. In addition, they are prepared by a gentle immobilization method in an elastic PVA matrix known for its low toxicity, mechanical and good long-term stability and low biodegradability (Ding & Vorlop, 1995; Parascandola, Branduardi, & De Alteriis, 2006; Rebroš, Rosenberg, Mlichová, & Krištofíková, 2007). LentiKats® have been succesfully used for entrapment of cells, e.g. *Zymomonas mobilis* (Rebroš, Rosenberg, Stloukal, & Krištofíková, 2005), yeasts (Bezbradica, Obradovic, Leskosek-Cukalovic, Bugarski, & Nedovic, 2007) and enzymes, e.g. β-galactosidase (Grosová, Rosenberg, Rebroš, Šipozc, & Sedláčková, 2008) and glucoamylase (Rebroš, Rosenberg, Mlichová, Krištofíková, & Palúch, 2006).

The aim of our work was to examine the potential application of the non-aggressive LentiKats® technique for enzyme and cells immobilization in poly(vinylalcohol) in the simultaneous process of D-galactose production.

2. Materials and methods

2.1. Materials

Saccharomyces oviformis RIVE V 10-25-23 was obtained from the CCY collection of the Slovak Academy of Sciences in Bratislava. Microorganisms were stored at 4 °C, pH 6.8, on the medium,

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consisting of 20 g l^{-1} of glucose (Mikrochem, Slovakia), 10 g l^{-1} of yeast extract (Biospringer, France), 5 g l^{-1} of mycological peptone (Oxoid, England) and solidified with agar (20 g l^{-1}, Oxoid, England).

Saccharomyces cerevisiae was obtained from the industrial distillery, Kolín, Czech Republic. Microorganisms were stored at 4 °C, pH 6.8, on the propagation medium (described in Section 2.2) solidified with agar (20 g l^{-1}, Oxoid, England).

Aspergillus oryzae β-galactosidase G5160 was obtained from Sigma (USA, lyophilised powder, 8 U mg^{-1}).

Kluyveromyces lactis β-galactosidase 3000 L HP-G (Lactozym®) was obtained from Novozymes (Germany, liquid form, 85 U mg^{-1}). In this work, one unit (U) was defined as the amount of enzyme able to convert 1 g of lactose per hour to form glucose and galactose at 30 °C and pH 4.5 (*A. oryzae* enzyme) or pH 6.5 (*K. lactis* enzyme). The specific enzyme activity was defined as the enzyme activity per 1 ml of free enzyme (U ml^{-1}) or per 1 g of immobilized enzyme (U g^{-1}). The relative activity was calculated as the ratio of actual to maximum activity.

Poly(vinylalcohol) (PVA 17-99), and polyethylene glycol were provided by LentiKats Inc. (Praha, Czech Republic). Food lactose was delivered by Milei (Germany). Other chemicals were of analytical grade and were delivered by Lachema Ltd. (Brno, Czech Republic) or Mikrochem Ltd. (Pezinok, Slovakia).

2.2. Media

2.2.1. Production medium

The medium consisted of 200 g l^{-1} of lactose (Milei, Germany), 1 g l^{-1} of yeast extract (Biospringer, France), 1 g l^{-1} of $(NH_4)_2SO_4$, 1 g l^{-1} of KH_2PO_4, 0.5 g l^{-1} of $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 4.5 or 6.5 (by 2 M KOH).

2.2.2. Inoculation and propagation medium

The medium consisted of 100 g l^{-1} of glucose (Mikrochem, Slovakia), 5 g l^{-1} of yeast extract (Biospringer, France), 5 g l^{-1} of $(NH_4)_2SO_4$, 1 g l^{-1} of KH_2PO_4, 0.5 g l^{-1} of $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 6.5.

All media were autoclaved.

2.3. Preparation of biomass for immobilization

Inoculum was prepared in two steps. At first, 50 ml of inoculation medium (100 ml Erlenmeyer flask) were inoculated with *S. cerevisiae* and cultivated statically (24 h) at 30 °C. The biomass was then used as inoculum (5% v/v) in 250 ml of inoculation medium (500 ml Erlenmeyer flask) and cultivated under the same conditions.

Prepared inoculum (5% v/v) was grown in 1.5 l of inoculation medium (3 l Erlenmeyer flasks) statically (24 h) at 30 °C. Cells were harvested in the exponential phase (OD_{600} 2) by centrifugation (1200*g*, 15 min) and suspended in a solution of NaCl (8.5 g l^{-1}).

2.4. Immoblization of enzymes and cells

The immobilizations of *S. cerevisiae* cells and β-galactosidases were performed on the pilot-scale by LentiKat's Inc. (www.lentikats.eu, see also Ding and Vorlop (1995)). Biomass (0.7 g of the cells suspended in 50 ml of solution of NaCl (8.5 g l^{-1})) or 50 ml of enzyme (*A. oryzae* β-galactosidase (0.3 g ml^{-1}) was dissolved in 0.1 M acetate buffer, pH 4.5, mixed with 1 l of PVA gel (100 g l^{-1}). The mixture was extruded through thin nozzles onto a hard surface and dried in a laminar airflow cabinet to 30% of the initial mass. Solid gel particles (LentiKats®) were swollen in stabilizing solution for 45 min according to the manufacturer's instructions.

2.5. Propagation of cells in LentiKats®

Biomass (*S. cerevisiae*) immobilized in LentiKats® (described above) was propagated in a stirred-tank bioreactor filled with 3 l of propagation medium and approximately 400 g of immobilizates at 30 °C with agitation (200 rpm). When the glucose utilization reached 90%, medium was separated and LentiKats® were washed with distilled water and transferred into fresh propagation medium. After eight cycles, the immobilizates, with biomass concentration of 160 mg in 1 g of LentiKats®, were used in all experiments.

2.6. Batch fermentations on hydrolyzed lactose

Erlenmeyer flasks (500 ml) containing of 270 ml of production medium with hydrolyzed lactose (200 g l^{-1}, prepared externally, reaching almost 80% of conversion by immobilized *K. lactis* β-galactosidase) were inoculated with 10% (v/v) of inoculum (*S. cerevisiae* or *S. oviformis*, both prepared as described in preparation of biomass for immobilization part). Fermentations were carried out at 30 °C and pH 6.5 with addition of 0.4 g of $CaCO_3$ at the beginning of the processes. The reaction mixture was agitated (130 rpm) with a magnetic stirrer. Experiments were duplicated.

2.7. SSF with free cells and enzyme

A fermentor (1.3 l) containing 0.8 l of production medium (lactose 200 g l^{-1}) was inoculated with *S. cerevisiae* inoculum (10% v/v, prepared as described in preparation of biomass for immobilization part) and *K. lactis* β-galactosidase (0.2%). SSF was carried out at 30 °C, pH 6.5 (automatic addition of 2 M KOH) and agitation of 200 rpm. Experiments were duplicated.

2.8. Repeated batch SSF

A fermentor (1.3 l) containing 0.8 l of production medium (lactose 200 g l^{-1}) was inoculated with enzyme and *S. cerevisiae* in free or immobilizate forms (specified in the graph legends). Fermentations were carried out at pH 4.5 (experiments with *A. oryzae* β-galactosidase) or 6.5 (experiments with *K. lactis* β-galactosidase, maintained by automatic addition of 2 M KOH), temperature of 30 °C and gently stirring (200 rpm). When lactose hydrolysis reached 90–100%, and almost all glucose was utilized, fermentation was stopped. After each batch cycle, 90% of the medium was separated and the rest (10%) served as inoculum for the next batch run (for the experiments with immobilized yeast, whole medium was separated).

2.9. Analytical assays

Free cells biomass in the medium was calculated from the correlation curve between the O.D. at 600 nm and dry cells weight. Final galactose amount was calculated from final galactose concentration in the reaction broth multiplied by the reaction volume. Concentrations of lactose, glucose, galactose and ethanol were determined by HPLC with a refractive index detector K-2301 (Knauer, Germany), Ionex column (Watrex 250 × 8 mm), Polymer IEX 8 μm H form (Watrex, Czech Republic) with 9 mM H_2SO_4 as mobile phase, at 50 °C and at flow rate 0.7 ml min^{-1}.

3. Results and discussions

3.1. Simultaneous saccharification and fermentation (SSF)

The process for galactose production from lactose consists of three main steps: hydrolysis of lactose, separation of D-galactose

Table 1
Parameters of free and immobilized β-galactosidases.

Source of enzyme	*T* optima[a] (°C)	pH optima[b]	Recovered activity[c] (%)
A. oryzae	55	4.5	32
K. lactis	50	6.5	20

[a] *T* optima-temperature optima for free enzymes.
[b] pH optima for free and immobilized enzymes (the same value).
[c] Recovered activity of enzyme after immobilization to LentiKats®.

from a mixture of galactose and glucose and eventually purification of D-galactose (Molle & Kempener, 2000). The work deals with the first two above-mentioned steps. In the first step, 200 g l^{-1} of lactose were enzymatically hydrolyzed by β-galactosidase in the free and immobilized form, yielding a mixture of D-galactose and D-glucose. Two different enzymatic preparations of β-galactosidase were tested. Their specifications are summarized in Table 1. In the second step, galactose was separated from the mixture of two hexoses by selective fermentation of the glucose to ethanol using yeasts. The galactose may then be easily removed, using known purification techniques (Rosenberg, 2000). Two different yeast strains were tested for this purpose, namely industrial strain *S. cerevisiae* and *S. oviformis* V-10-25-23. The yeasts were used for fermentation of glucose from hydrolyzed lactose (200 g l^{-1}) and compared. Both of them had high selectivity for conversion of D-glucose to ethanol while the production of organic acids and other by-products was minimal. But the consumption of D-glucose was 1.3-times faster when using *S. cerevisiae* than when using *S. oviformis,* under identical conditions. Therefore, the following experiments were undertaken with the strain *S. cerevisiae*.

D-Galactose production was achieved as a process of simultaneous saccharification and fermentation (SSF), where hydrolysis of lactose and fermentation of generated glucose were carried out in one reactor. The process of SSF offers several advantages, such as decrease of investment for hydrolysis and fermentation vessels and increase of hydrolysis rate, due to continuous glucose reduction and therefore reduction of fermentation time (Eklund & Zacchi, 1995). The optimal conditions for the SSF process with *K. lactis* β-galactosidase and *S. cerevisiae* were 30 °C and pH 6.5. The temperature, 30 °C, was determined by the optimal temperature for the fermentation process by the yeast. Moreover, although the optimal temperature for hydrolysis of lactose by *K. lactis* β-galactosidase is 50 °C, the long-term stability of the enzyme is lower at higher temperatures. pH value 6.5 was determined by the maximum activity of enzyme used for the lactose hydrolysis process. Under these conditions, the enzyme still showed high activity (40% of initial activity at optimal temperature) and the rate of the fermentation was only gently decreased. The typical SSF batch conversion with free *K. lactis* β-galactosidase (0.2%) and free microorganism (10%) acting on lactose (200 g l^{-1}) was completed in 31 h and 2.9 g l^{-1} h^{-1} of galactose productivity was achieved (Fig. 1). The hydrolysis of lactose reached 95%, after this point, the reaction was significantly inhibited by product formation. The overall duration of the process was only 31 h compared to 41.5 h, which included the time of separate hydrolysis of lactose (200 g l^{-1}) at 50 °C (9 h), cooling (1.5 h) and fermentation by the yeast at 30 °C (31 h). Moreover, lactose hydrolysis, with fermentation of glucose, decreases the product inhibition of the enzyme, because the accrued glucose was simultaneously utilized by the yeast.

3.2. SSF with immobilized enzyme and free S. cerevisiae

Main limits of the above-described process are of an economical nature, which greatly increase the production costs: the cost of the enzyme and the need to purify the starting material. To overcome the first problem, immobilized enzyme was used. β-Galactosidases

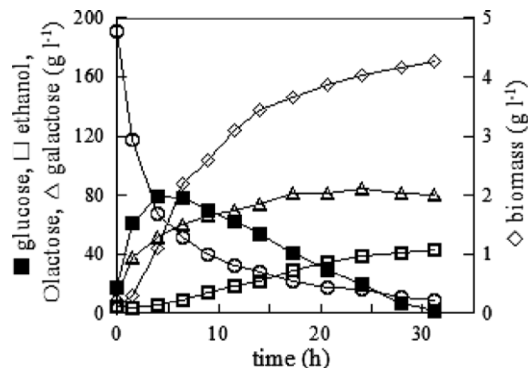

Fig. 1. Batch SSF process with free *K. lactis* β-galactosidase and free *S. cerevisiae* on lactose (200 g l^{-1}).

were immobilized to PVA hydrogel in form of LentiKats®. The method of immobilization was chosen because of the good properties and excellent results achieved with its application in previous trials (Bezbradica et al., 2007; Grosová et al., 2008; Rebroš et al., 2005, 2007). Although, the enzyme lost activity after immobilization (Table 1), repeated use of immobilizates can compensate for this. Repeated batch cycles were performed with immobilized *K. lactis* β-galactosidase and 10% *S. cerevisiae* inoculum on lactose (200 g l^{-1}). After each batch cycle (when lactose was hydrolyzed to about 95%), 90% of the medium was separated and the remaining (10%) was inoculum for the next batch run. After 20 batch runs, enzyme still remained at about 50% of the initial activity (Table 2). Almost 20% of activity was lost at the first run, most likely due to the adsorption of the free cells of yeast on the surface of LentiKats® capsules, which limited the access of substrate to entrapped enzyme. The adsorption of microorganisms was confirmed by microscopic observation. A similar decrease (50%) in galactose productivity was observed (from 6 to 3 g l^{-1} h^{-1}). Nearly 1.4 kg of galactose was prepared during 466 h period of SSF with immobilized enzyme, which represented 85% of the theoretical yield of galactose (Fig. 2, Table 2).

An experiment was also performed with immobilized *A. oryzae* β-galactosidase under the same conditions at pH 4.5. Although the entrapped enzyme lost about 20% of initial activity at the first run (as in the above-mentioned experiment), enzyme activity of the next 14 repeated batch runs was stable (Table 2). Presumably, the great long-term operational stability of the enzyme is related to its stability in the PVA matrix, which should be caused by interaction of the PVA gel with the enzyme. Compared to *K. lactis* β-galactosidase, the immobilized *A. oryzae* β-galactosidase showed great operational stability, but 1.6-times lower galactose productivity at the end of the process (1.9 g l^{-1} h^{-1}). This was caused by strong inhibition by galactose formation, which led to marked deceleration of lactose hydrolysis after 60% of conversion, and it reached a maximum conversion 91%. Therefore, the amount of produced galactose was only 0.9 kg during 466 h of the process, which corresponds to 75% of the theoretical yield (Fig. 2, Table 2). Besides great stability, the next advantage of *A. oryzae* β-galactosidase is the elimination of contamination risk due to the low pH (4.5) applied in the process of SSF. Both of immobilized enzymes had their own benefits and their selection depends mainly on the substrate and fermentative conditions for the process of D-galactose manufacture.

3.3. SSF with immobilized S. cerevisiae

To increase the production of D-galactose, with elimination of the effect of free yeast adsorption, immobilized *S. cerevisiae* cells were also used in the process of SSF with immobilized *K. lactis*

Table 2
Parameters of repeated batch SSF with β-galactosidase and *S. cerevisiae* in free or immobilized form.

Source of enzyme	Type and amount of enzyme	Type and amount of yeast	Number of batch runs	Produced galactose[a] (kg)	Productivity[a] ($g\,l^{-1}\,h^{-1}$)	Drop of enzyme activity[a] (%)	Duration of experiment (h)
K. lactis	Immobilized, 50 g	Free, 10% (v/v)	20	1.4	3	50	466
A. oryzae	Immobilized, 60 g	Free, 10% (v/v)	15	0.9	1.9	20	466
K. lactis	Immobilized, 60 g	Immobilized, 40 g	23	1.4	4.1	20	340
K. lactis	Free, 3 ml	Immobilized, 80 g	18	1.2	6.4	–	203

[a] The value of parameter at the end of the experiment.

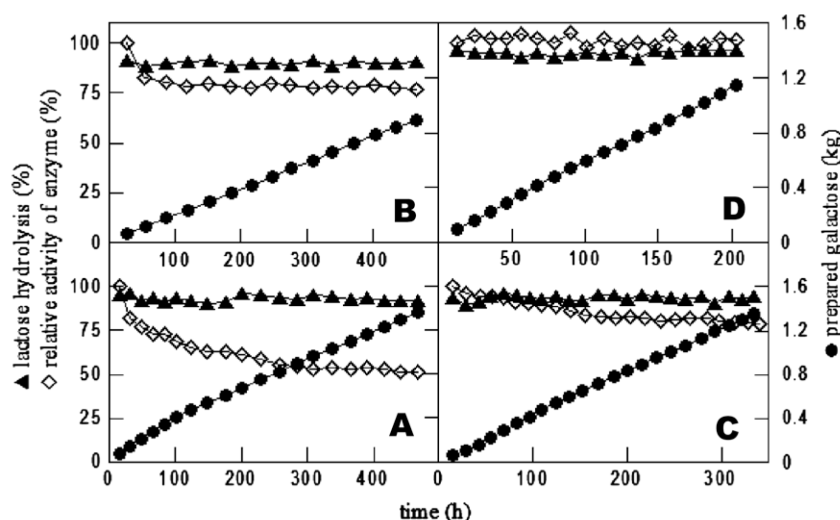

Fig. 2. Repeated batch SSF with (A) immobilized *K. lactis* β-galactosidase (50 g) and free *S. cerevisiae* (10% v/v inoculum), (B) immobilized *A. oryzae* β-galactosidase (60 g) and free *S. cerevisiae* (10% v/v inoculum), (C) immobilized *K. lactis* β-galactosidase (60 g) and immobilized *S. cerevisiae* (40 g) and (D) free *K. lactis* β-galactosidase (3 ml) and immobilized *S. cerevisiae* (80 g) on lactose (200 $g\,l^{-1}$). 100% relative activity corresponds to 0.23 and 0.11 $U\,g^{-1}$ of specific enzymatic activity for immobilized β-galactosidase from *K. lactis* and *A. oryzae*, respectively, and 4.3 $U\,ml^{-1}$ for free *K. lactis* β-galactosidase. The values of all parameters correspond to those obtained at the end of each fermentation run.

β-galactosidase. In addition, immobilization of the yeast biomass allowed us to reach higher concentrations in the bioreactor, resulting in increase of the ethanol production rate. Compared to the free yeast process, the same amount of galactose (1.4 kg) was obtained only in 340 h of the fermentation (Fig. 2) and with 1.4-times higher galactose productivity (4.1 $g\,l^{-1}\,h^{-1}$) (Table 2). Moreover, co-immobilization of yeasts and enzyme increased the stability of the enzyme activity and, after 23 cycles of repeated batch hydrolyses, immobilized enzyme retained 80% of the initial activity. Entrapped enzyme and yeast were stable during the entire experiment without any changes in mechanical quality of the matrix. Alternatively, free β-galactosidase in SSF with entrapped *S. cerevisiae* can be used in this process. The production capabilities of the yeast were stable in a series of 18 repeated batch fermentations and no changes in volumetric productivity of immobilizates were observed (6.4 $g\,l^{-1}\,h^{-1}$) (Fig. 2, Table 2). However, the combination of free β-galactosidase and immobilized cells, did not solve the problem of the expense associated with the high price of enzymes.

4. Conclusions

The present work provides a novel approach to the production of D-galactose from lactose using immobilized enzymes and yeasts to poly(vinylalcohol) (PVA) hydrogel in form of LentiKats® particles. The process, consisted of two steps (lactose hydrolysis and selective fermentation of glucose), achieved simultaneously in one bioreactor. Compared to the separate processes, SSF saves the energy requirement for pre-hydrolysis and reduces the overall duration of the process (from 41.5 to 31 h). The use of immoblized enzymes did not increase the galactose productivity, but repetitive use of immoblized enzymes results in reduction of investments in the enzyme. From the two tests, *A. oryzae* β-galactosidase showed excellent operational stability, retaining 80% of initial activity during 466 h of repeated batches of SSF with free yeast, but lower galactose productivity (1.9 $g\,l^{-1}\,h^{-1}$). Under the same conditions, *K. lactis* β-galactosidase retained 50% of initial activity and galactose productivity of 3 $g\,l^{-1}\,h^{-1}$. Compared to the free enzyme, 1.6-times more D-galactose (1.4 kg) was prepared with twice the amount of enzyme in the immobilized form; furthermore, the process can be operated for a longer time period.

Further improvement of the process was achieved by the SSF process with both, enzyme (from *K. lactis*) and cells immobilized in PVA hydrogel. Almost 30% smaller loss of enzyme activity, and hence 30% higher volumetric galactose productivity, were observed for the 23 repeated batch cycles needed for production of 1.4 kg D-galactose. LentiKats® particles of yeast showed high mechanical and fermentative stability, since they remained in use for 340 h of operation without change of cell activity, shape or size of capsules.

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